Journal of Medical Virology 54:140–144 (1998)

A Clinical and Epidemiological Study of Human Parvovirus B19 Infection in Fetal Hydrops Using PCR Southern Blot Hybridization and Chemiluminescence Detection

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Ninety-eight samples from 80 cases of spontaneous abortions after fetal death or hydrops fetalis from 12,000 pregnant women were examined using PCR. DNA was extracted from amniotic fluid, fetal blood, ascitic fluid and fetal biopsies or placenta specimens using QIA amp kits (QIAGEN). A 270-bp length fragment located within the B19 gene NS1 was amplified using PCR followed by electrophoresis and southern-blot hybridization assay using a horseradish peroxidase-labelled probe and chemiluminescence detection. This assay was able to detect 1 to 10 DNA copies in a 10 µl sample. Parvovirus B19 was identified in 11 cases (14% of fetal hydrops; 1 case for 1,100 pregnancies). Amniotic fluid was the most common and reliable sample to assess the diagnosis. Gestational age ranged from 17 to 28 weeks (mean 23 weeks). IgM antibodies were detected in 3 maternal sera, 2 patients of which reported an exposure to B19 infection during pregnancy. In 2 cases, intrauterine blood transfusions led to the cessation of symptoms and to birth of normal babies. ***J. Med. Virol. 54:140–144, 1998.***

KEY WORDS: human parvovirus B19; hydrops fetalis; PCR; chemiluminescence detection

INTRODUCTION

Human parvovirus B19 was first discovered in blood donors by Cossart et al. [1975]. Infection is associated with erythema infectiosum or fifth disease, acute arthritis, aplastic crisis in patients with hemolytic anaemia and with hydrops fetalis, spontaneous abortion or stillbirth during pregnancy [Brown et al., 1990; Leruez and Morinet, 1994; Soulié, 1995]. However, fetal abnormalities represent rare events [Weiland et al., 1987]. Women with a current parvovirus B19 infection during pregnancy may transmit the virus to the fetus during the viremic phase. Infection of the fetus may cause damage of the red precursor cells resulting in severe anemia, congestive heart failure and generalised oedema. Early in pregnancy, parvovirus infection may induce spontaneous abortion; during the second trimester, parvovirus infection may cause non-immune hydrops fetalis and/or intrauterine death; at term parvovirus infection may result in stillbirth. About 4% of the 30 to 50% non-immune women acquire the disease during pregnancy; 30% may transmit the infection to the fetus with only 10% resulting in fetal damage [Woernle et al., 1987; Gratacos et al., 1995].

Diagnosis of parvovirus B19 infection is based either on serological assays or on viral detection by immunological methods or DNA detection using hybridization assays. Parvovirus particles have been demonstrated in clinical samples by direct or immunoelectron microscopy and located in erythroblast cells [Clewley et al., 1987; Caul et al., 1988; Field et al., 1991]. B19 capsid antigens have also been detected in blood and in tissue extracts using immunoassays [Anderson et al., 1986]. Anti B19 IgM and IgG antibodies can be detected both in fetal and maternal blood samples using indirect immunofluorescence or enzyme immunoassays [Brown et al., 1989, 1990; Morinet et al., 1989, 1991; Yaegashi et al., 1989; Schwartz et al., 1991; Bruu and Nordbo, 1995; Cohen and Bates, 1995; Sloots and Devine, 1996]. However, these techniques frequently lack sensitivity and specificity and are not applicable for the diagnosis of B19 infection of the fetus following maternal infec-

Contract grant sponsor: The Centre Hospitalier Universitaire de Lille; Contract grant number: 93-11.

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Accepted 6 October 1997

tion during pregnancy. More recently B19 DNA detection has been evaluated using PCR assays with or without subsequent southern-blot hybridisation and it has been concluded that these techniques are the most sensitive to detect the virus in clinical samples [Clewley, 1989; Azzi et al., 1990; Koch and Adler, 1990; Nascimento et al., 1991; Torok et al., 1992; Carriere et al., 1993; Cassinotti et al., 1993; Erdman et al., 1994; Gallinella et al., 1994].

We report the results of a retrospective study on the diagnosis of parvovirus infection during pregnancy using a PCR protocol followed by a southern-blot hybridisation assay using a horseradish peroxidase-labelled probe and chemiluminescence detection.

MATERIALS AND METHODS

Patients and Clinical Specimens

During three years from 1994 to 1996, clinical specimens from 80 cases of fetal hydrops with or without fetal loss from a total of approximately 12,000 pregnant women were submitted to the Virology Laboratory at the Centre Hospitalier Universitaire of Lille (France) and tested for parvovirus B19 infection using DNA detection by PCR. Maternal and fetal sera were examined for parvovirus B19 IgM and IgG antibodies. A total of ninety-eight specimens were tested for parvovirus B19 DNA including 55 amniotic fluids, 13 fetal bloods, 7 placental biopsies, 3 ascitic fluids and 20 organ biopsies (heart, $n = 10$; liver, $n = 5$; spleen, $n = 3$; lung, $n = 1$ and kidney, $n = 1$). Also included were 5 amniotic fluids from patients with evidence of disease other than parvovirus infection, including 2 positive for cytomegalovirus infection, 3 for non-infectious disease, and sera from 5 patients with hemolytic anaemia not caused by parvovirus B19. Information regarding gestational age, exposure to B19 infection during pregnancy and pregnancy outcome were collected.

IgM and IgG Assays

From 1994 and 1995, detection of specific B19 IgM and IgG antibodies in sera was performed using MACRIA and GACRIA (IgM and IgG antibody capture radioimmunoassays, CNTS, Paris, France) [Morinet et al., 1991] and since 1995 using Biotrin Parvovirus B19 enzyme immunoassays (Parvo B19 EIA, Biotrin International, Lyon France) according to the manufacturer's instructions.

DNA Extraction

For amplification of serum, amniotic fluid, ascitis, EDTA treated blood and EDTA treated bone marrow samples, DNA was extracted using QIA Amp blood kit (QIAagen, Hilden, Germany). DNA from small fragments of placental tissue or organ specimens was extracted using QIA Amp Tissue kit (QIAgen, Hilden, Germany). Briefly, after a first step of lysis, the sample was bound to a QIAamp spin column. The column was then washed twice and the purified DNA eluted from the column in distilled water preheated at 70°C according to the manufacturer's recommendations.

Amplification Assays

A 270 bp fragment located within the NS1 coding sequence was amplified. The validated sequences of the primers are: 5′ GTT AAC ATC CTA ACA TGG A (sense nucleotide 422 to 440) and 5′ GTA ACC ACA TGA ATA TGA TA (antisense nucleotide 692 to 673) (Genset, Paris, France). The reaction mixture contained 5μl sample, 10 μl 10x buffer (100 mM tris HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 1 mg/ml gelatin) (Boehringer Mannheim, Germany), 0.8 μl of a mixture containing 25 mM each deoxynucleotide triphosphate (Boehringer Mannheim, Germany), 2 μl (200 ng) each primer, 0.5 μl $MgCl_2$ (50 mM) and 71.2 μl water. The reaction mix was overlaid with mineral oil. After an initial denaturation step at 94°C for 10 min, 35 cycles were conducted (denaturation step at 94°C for 1 min, annealing step at 55°C for 1 min, extension step at 72°C for 1 min), followed by an additional step of extension at 72°C for 5 min in a DNA thermal cycler 480 (Perkin Elmer, Saint Quentin en Yvelines, France). A pBR322 plasmid containing the B19 virus full-length genome cloned into the EcoRI site of pBR322 [Morinet et al., 1986] and a B19 positive serum were used as positive controls. Positive and negative controls, cytomegalovirus and herpes simplex virus DNA extracts and globin gene were conducted according to the same protocol.

Detection of B19 Amplified Sequences

The products of the amplification were electrophoresed in 2% agarose gel in Tris borate buffer containing ethidium bromide and viewed under UV light. Southern transfer and hybridisation with a horseradish peroxidase labelled B19 probe (ECL Kit, Amersham, England) (5′ GAC TGT GCT AAC GAT AAC TGG TGG TGC TCT TTA CTG GAT T, nucleotide 481 to 520) were performed on Hybond N membrane (Amersham, England). Before transfer, the gels were soaked in 0.5 M NaOH, 1.5 M NaCl for 25 min, followed by two washes for 10 min in buffer containing 1.5 M NaCl, 0.5 M Tris HCl pH 7.5. After transfer, the membrane was prehybridised at 42°C for 1 hr (Bioprobe system) in hybridization solution (0.125 ml/cm2, ECL Gold Buffer, Amersham) with shaking, and hybridized overnight at 42°C with the labelled probe. The filter was washed twice in buffer (0.5 × SSC, pH 7.0, 0.4% SDS with urea 6 M) at 42°C for 20 min followed by two washes in the same buffer without urea at 42°C for 5 min, and the hybridized probes was revealed using a biochemiluminescent assay according to the manufacturer's instructions (ECL Detection Kit, Amersham, England) and exposed to an X-ray film for 30 min.

RESULTS

Compared with saline buffer and phenol-chloroform extractions, the use of QIAamp spin column provided the best standardised way to extract and purify DNA without inhibitors for reliable PCR and southern-blotting (data not shown). The specificity of the amplification reaction was verified with a molecularly cloned

TABLE I. Clinical and Virological Status of 11 Parvovirus B19 Infections During Pregnancy Associated with Fetal Hydrops

Cases	Gestational age	Anti B19 IgM/IgG		PCR B19 DNA positive samples	Clinical findings and outcome
		Maternal serum	Fetal serum		
1	28	−/+	−/+	Heart	1 BT Fetal death
2	25	−/+	−/+	Heart Spleen Liver Placenta	Spontaneous abortion
3	17	+/+	−/+	Heart Serum Liver Spleen Lung	Spontaneous abortion
4	21	−/+	−/+	Serum	3 BT Birth of normal baby
5	24	−/+	−/+	Amniotic fluid	2 BT Birth of normal baby
6	25	−/+	−/+	Amniotic fluid	Spontaneous abortion
7	21	+/+	ND	Amniotic fluid Placenta	1 BT Spontaneous abortion
8	28	ND	ND	Spleen Liver Heart Placenta	Fetal death
9	26	ND	ND	Placenta	Fetal death
10	23	+/+	ND	Amniotic fluid	Fetal death
11	22	ND	ND	Amniotic fluid	Surviving fetus without BT

Anti B19 IgM and IgG antibodies were detected using MACRIA and GACRIA for patients 1 to 6 and using EIA for patients 7 and 10.B19 DNA was detected using a PCR assay followed by southern-blotting hybridization test and chemiluminescence detection.
ND: not done; BT: blood transfusion.

B19 DNA and a B19 DNA positive serum. After 35 cycles of PCR, 1 to 10 B19 DNA copies were detectable by southern blotting hybridization followed by a chemiluminescence assay (data not shown). No PCR product was obtained with all the control samples and with other extracted virus DNA tested. B19 DNA was detected in 22 samples (5 amniotic fluids, 4 placental biopsies, 2 sera, 11 organ biopsies) corresponding to 11 cases of parvovirus B19 infection from 80 cases of fetal hydrops (14%). They represent 1 case per 1,100 pregnancies. Gestational age ranged from 17 to 28 weeks (median = 23). All the women were multigravida and multipara. Only two women reported having been directly exposed during pregnancy to a child with a rash identified as erythema infectiosum four weeks earlier (cases 3 and 5). Virus inclusions were shown in the nucleus of erythroid precursor cells in 3 fetuses (cases 2, 6 and 9). B19 IgG antibodies were detected in both fetal and maternal sera in all the cases, whereas B19 IgM antibodies were reported in 3 cases only in the mother. In 2 cases, intrauterine blood transfusions led to the cessation of symptoms and to birth of normal babies (Table I).

DISCUSSION

In this study, 98 maternal-fetal samples from 80 pregnant women with fetal hydrops were examined for parvovirus B19 infection by PCR and southern blot hybridization with chemiluminescence detection which allowed the identification of 11 cases of parvovirus B19 fetal hydrops.

Currently there is no tissue culture system for detecting human parvovirus B19 or commercially available antigen detection kit. Parvovirus B19 diagnosis is based on either detection of parvovirus B19 specific antibodies or B19 DNA. Antibody detection can be performed by immunofluorescence assays on slides containing cells expressing parvovirus structural protein VP1 [Brown et al., 1990] or by commercial enzyme immunoassays using purified whole antigen [Yaegashi et al., 1989], recombinant protein [Morinet et al., 1989; Morinet et al., 1991] or synthetic peptide antigen (VP1 and VP2) [Schwartz et al., 1991]. Several commercial kits have been compared and have shown concordant results in parvovirus B19 infected patients [Bruu and Nordbo, 1995; Cohen and Bates, 1995; Sloots and Devine, 1996] with a specificity range from 97 to 100% and a sensitivity range from 79 to 99%. However, in our patients, IgM antibodies were detected only in the sera of 3 mothers and all the fetal sera were B19 IgM negative. A lack of IgM antibodies can occur especially in the fetus and in patients who have eliminated rapidly the virus [Cassinotti et al., 1993]. Moreover, false positive results can be obtained in patients with an IgM response to rubella, toxoplasmosis, or cytomegalovirus infections [Cohen and Bates, 1995; Sloots and Devine, 1996]. Because of the absence of a relationship between IgM antibody detection and maternal infection and its

low-level of specificity, the B19 IgM assays are not recommended for determining maternal and fetal infections during pregnancy. Although an alternative approach for determining maternal infection could be to test for IgG seroconversion using the booking serum collected for screening tests since the beginning of pregnancy [Cohen and Bates, 1995], serological assays are not reliable enough. Parvovirus B19 primarily replicates in erythroid precursor cells and virus inclusions can be detected in these cells in fetal biopsies; tissues or body fluids containing such cells are therefore suitable for B19 DNA detection. Dot-blot hybridisation assays using a ^{32}P labelled probe or a digoxigenin labelled-probe showed a sensitivity of 0.1 to 0.2 pg B19 DNA for 10 μl sample [Azzi et al., 1990; Durigon et al., 1993; Gallinella et al., 1994; Zerbini et al., 1990]. The sensitivity of B19 DNA detection was increased using PCR or nested-PCR and identification of amplification products by agarose gel electrophoresis stained with ethidium bromide (sensitivity from 10 to 200 genomes for 10 μl) [Cassinotti et al., 1993; Durigon et al., 1993], or by southern-blotting followed by autoradiography with a ^{32}P labeled probe (sensitivity from 10 to 100 genomes for 10 μl) [Carriere et al., 1993; Clewley, 1989; Koch and Adler, 1990] or by enzyme immunoassay with a digoxigenin labeled-probe (sensitivity from 3 to 30 genomes) [Erdman et al., 1994]. Concordant results for amplification of a B19 parvovirus non-structural protein coding sequence (NS1) or of viral capsid protein coding sequences (VP1,VP2) have been reported previously [Koch and Adler, 1990]. It was shown that the use of a PCR followed by southern-blotting and chemiluminescence detection was able to detect 1 to 10 DNA copies in 10 μl of sample and was a very sensitive specific and rapid tool for the diagnosis of parvovirus B19 infection during pregnancy. We demonstrated also that QIA amp spin column procedure for DNA extraction was rapid, practical and reliable for routine screening of parvovirus DNA in large numbers of clinical specimens.

Because there is little information about exposure to B19 infection during pregnancy and because primary infection is commonly asymptomatic, the diagnosis of parvovirus fetal hydrops is generally discovered only by echography. Parvovirus B19 fetal hydrops appears to be a major cause of fetal death. The risk of fetal death following maternal B19 infection during pregnancy is 10% (8/80) based on our results and is in agreement with the estimation of Cartter et al. [1991]. It represents a frequency of 1 case for 1,100 pregnancies which is higher than this reported by Gratacos et al. (1 case for 2,000 pregnancies) [1995] and could be explained by the focusing of these patients in an obstetrician expert centre. Parvovirus B19 infection was responsible for 27% of non-immune hydrops which happen at the end of the second trimester of the pregnancy (23 weeks in our study) These results are in agreement with those of Brown et al. [1984], Woernle et al. [1987], Caul et al. [1988], and Anand, Anderson, Naides and Morey cited by Soulié [1995] (19 to 24 weeks). At this time, when haemolysis is moderate, repeated intrauterine blood transfusions will benefit some fetuses by reducing oedema and symptoms and led to the birth of normal babies as achieved in cases 4 and 5 [Fairley et al., 1995]. Although the true incidence of B19 embryopathy is underestimated since many fetal B19 infections do not present as fetal hydrops, our results suggest that the frequency of parvovirus B19 infection during pregnancy with hydrops fetalis is not a rare event and that a B19 DNA amplification using PCR followed by southern-blotting hybridization revealed with a chemiluminescence assay is available for routine use. It was also concluded that amniotic fluid is the most appropriate sample for B19 DNA detection and the easiest to test. Moreover, this early diagnosis may induce an appropriate treatment using in utero blood transfusions and permit the birth of normal babies.

ACKNOWLEDGMENTS

We are grateful to Professor Morinet for kindly providing the plasmid.

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